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(71) Applicant *(for all designated States except US)*: DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(71) Applicant *(for US only)*: PEDERSEN, Rolf, Frost (heir of the deceased inventor) [DK/DK]; Hedevej 9, DK-9280 Store Vorde Mou (DK).

(72) Inventor: PEDERSEN, Helle, Frost (deceased).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KREIBERG, Jette, Dina [DK/DK]; Dueholm 18, DK-4000 Roskilde (DK). LUND, Marianne [DK/DK]; Vestertro 35, DK-8464 Galten (DK).

(74) Agents: HARDING, Charles, Thomas et al.; D Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

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(54) Title: PROMOTER FROM A PLANT ALPHA-AMYLASE GENE

(57) Abstract

A promoter is described that is capable of expresisng a GOI in any one of sprout and, preferably, stem tissue of a dicot plant. In particular, the promoter is a promoter for alpha-amylase.

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# Promoter from a plant alpha-amylase gene

The present invention relates to a promoter, including a construct and an expression vector comprising the same and a transformed cell comprising the same. In addition the present invention relates to a plant comprising the same.

It is known that it is desirable to direct expression of a gene of interest ("GOI") in certain tissues of an organism – such as a plant. For example, it may be desirable to produce crop protein products with an optimised amino acid composition and so increase the nutritive value of the crop. It may even be desirable to use the crop to express non-plant genes such as genes for mammalian products. Examples of the latter products include interferons, insulin, blood factors and plasminogen activators.

However, whilst it may be desirable to achieve expression of a GOI in certain tissues it is sometimes important (if not necessary) to ensure that the GOI is not expressed in other tissues in such a manner that detrimental effects may occur. Moreover, it is important not to upset the normal metabolism of the organism to such an extent that detrimental effects occur. For example, a disturbance in the normal metabolism in a plant's leaf or root tip could lead to stunted growth of the plant.

CA-A-2006454 describes a DNA sequence of an expression cassette in which the potato tuber specific regulatory regions are localised. The expression cassette contains a patatin-gene with a patatin-gene promoter. The DNA sequence is transferred into a plant genome using *agrobacteria*. According to CA-A-2006454, the DNA sequence enables heterologous products to be prepared in crops.

One of the key plant enzymes is $\alpha$-amylase. $\alpha$-amylase participates in the pathway responsible for the breakdown of starch to reducing sugars in potato tubers.

Genes coding for $\alpha$-amylase in potato plants have been isolated and characterised. For example, see the teachings in EP-B-0470145.

In brief, α–amylase is encoded by a gene family consisting of at least 5 individual genes. Based on their homology the genes can be divided into two subfamilies – one of which is the type 3 amylase(s), the other of which is the type 1 amylase(s). The two groups of α–amylases are expressed differently, not only on the molecular level but also in different tissues of the potato plant.

In this regard, type 3 α–amylases are expressed in tubers, in sprouts and in stem tissue; whereas type 1 α–amylases are expressed in sprout and stem tissues, but not in tubers or roots or leaves.

The present invention seeks to provide a plant promoter that is capable of directing the expression of a gene of interest in specific tissues, or in just a specific tissue, of an organism, typically a plant.

According to a first aspect of the present invention there is provided a promoter comprising the sequence shown as Seq.I.D.No. 1 or a variant, homologue or fragment thereof.

According to a second aspect of the present invention there is provided a promoter comprising the sequence shown as Seq.I.D.No. 1 or a variant, homologue or fragment thereof but wherein at least a part of the promoter is inactivated.

According to a third aspect of the present invention there is provided a construct comprising the promoter according to the present invention fused to a GOI.

According to a fourth aspect of the present invention there is provided an expression vector comprising the promoter according to the present invention or the previous aspect of the present invention.

According to an fifth aspect of the present invention there is provided a transformation vector comprising the promoter according to the present invention or any one of the other previous aspects of the present invention.

According to a sixth aspect of the present invention there is provided a transformed cell comprising the promoter according to the present invention or any one of the other previous aspects of the present invention.

According to a seventh aspect of the present invention there is provided a transgenic organism comprising the promoter according to the present invention or any one of the other previous aspects of the present invention.

According to an eighth aspect of the present invention there is provided the use of a promoter according to the present invention to direct expression of a GOI in sprout or, preferably, stem cell, tissue or organ of a plant.

Other aspects of the present invention include methods of expressing or transforming any one of the expression vector, the transformation vector, the transformed cell, including *in situ* expression within the transgenic organism, as well as the products thereof. Additional aspects of the present invention include uses of the promoters for expressing GOIs *in vitro* (e.g. in culture media such as a broth) and *in vivo* (e.g. in a transgenic organism).

Preferably, in any one of the expression vector, the transformation vector, the transformed cell or the transgenic organism the promoter is present in combination with at least one GOI.

Preferably the transformation vector is derived from *agrobacterium*.

Preferably the promoter is stably incorporated within the transgenic organism's genome.

Preferably the transgenic organism is a plant. Preferably the plant is a dicot plant. More preferably, the plant is a potato plant.

A key advantage of the present invention is that the promoter having the sequence shown as Seq. I.D. No. 1 is able to direct expression of a GOI in sprout and stem tissue of a dicot, for example, a potato. The same is true for the variant, homologue or fragment

thereof. Preferably, the promoter directs expression of a GOI in stem tissue.

Further surprising however is the fact that the promoter sequence can be truncated and it can still express a GOI.

More surprising is the fact that the truncated promoter sequences can direct expression of a GOI in one specific tissue (i.e. sprout or stem, preferably stem) - rather than a combination of sprout and stem tissues.

In this regard, a truncated version of the promoter sequence shown as Seq. I.D. No. 1 can direct expression of a GOI in just stem tissue or just sprout tissue. The same is true for variants, homologues or fragments thereof.

Tissue specific expression, such as stem specific expression, is particularly advantageous for a number of reasons.

In particular, it can be used to express a GOI to give resistance against diseases that affect stem tissue. For example, a known disease is black-leg which specifically affects potato stems and can produce soft rot in tubers (25). To improve the resistance in a potato crop against black-leg or any other disease which affect potato stems, it is possible to combine with the promoter of the present invention with a GOI coding for, for example, a toxin against the organism *Erwinia carotovora* causing the black-leg disease.

Highly preferred embodiments of each of the aspects of the present invention do not include the native promoter in its natural environment.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression. The promoters of the present invention are capable of expressing a GOI.

In addition to the nucleotide sequences described above, the promoters of the present invention could additionally include conserved regions such as a Pribnow Box or a TATA

box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the GOI. For example, suitable other sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements – such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' leader sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97). The promoter of the present invention is sometimes refered to as the alpha-Amy 1 promoter or the Amy 1 promoter or the Amy 637 promoter.

In addition the present invention also encompasses combinations of promoters or elements. For example, a promoter of the present invention, which may be a stem specific promoter, could be used with a tuber specific promoter. Other combinations are possible. For example, the promoter of the present invention, which may be stem or sprout specific promoter, could be used with a root tissue promoter and/or a leaf tissue promoter.

The terms "variant", "homologue" or "fragment" include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence has the ability to act as a promoter in an expression system –such as the transformed cell or transgenic organism according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as a promoter. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology, more preferably at least 95%, more preferably at least 98% homology.

The term "inactivated" means partial inactivation in the sense that the expression pattern of the promoter of Seq. I.D. No. 1 is modified but wherein the partially inactivated promoter still functions as a promoter. However, as mentioned above, the modified promoter is capable of expressing a GOI in at least one (but not all) specific tissue of the promoter of Seq. I.D. No. 1.

Therefore with this particular aspect of the invention, the promoter having an inactivated portion can still function as a promoter (hence it is still called a promoter) but wherein the promoter is capable of expressing a GOI in one or more, but not all, of the tissues where a GOI is expressed by a promoter having the sequence shown as Seq. I.D. No. 1.

Examples of partial inactivation include altering the folding pattern of the promoter sequence, or binding species to parts of the nucleotide sequence, so that a part of the nucleotide sequence is not recognised by, for example, RNA polymerase. Another, and preferable, way of partially inactivating the promoter is to truncate it to form fragments thereof. Anoter way would be to mutate at least a part of the sequence so that the RNA polymerase can not bind to that part or another part.

Accordingly, for a preferred embodiment of the present invention there is provided a promoter comprising a nucleotide sequence corresponding Seq.I.D. No. 1, or a variant, homologue or fragment thereof but wherein the promoter is truncated. The term "truncated" includes shortened versions of the promoter shown as Seq. I.D. No. 1.

The term "construct" – which is synonymous with terms such as "conjugate", "cassette" and "hybrid" – includes a GOI directly or indirectly attached to the promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*–intron or the ADH intron, intermediate the promoter and the GOI. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In each case, it is highly preferred that the terms do not cover the natural combination of the wild type alpha amylase gene ordinarily associated with the wild type gene promoter and the wild type promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a plant cell into which it has been transferred. Various markers exist which may be used in, for example, plants – such as mannose. Other examples of markers include those that provide for antibiotic resistance – e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

The term "GOI" with reference to the present invention means any gene of interest. A GOI can be any nucleotide that is either foreign or natural to the organism (e.g. plant) in question.

Typical examples of a GOI include genes encoding for proteins and enzymes that modify metabolic and catabolic processes.

The GOI may code for an agent for introducing or increasing pathogen resistance.

The GOI may even be an antisense construct for modifying the expression of natural transcripts present in the relevant tissues.

The GOI may even code for a non-natural plant compound that is of benefit to animals or humans. For example, the GOI could code for a pharmaceutically active protein or enzyme such as any one of the therapeutic compounds insulin, interferon, human serum albumin, human growth factor and blood clotting factors. In this regard, the transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, for example, stem.

The GOI may even be a protein giving nutritional value to the plant as a food or crop. Typical examples include plant proteins that can inhibit the formation of anti-nutritive factors and plant proteins that have a more desirable amino acid composition (e.g. a higher lysine content than the non-transgenic plant).

The GOI may even code for an enzyme that can be used in food processing such as chymosin, thaumatin and alpha-galactosidase. Preferably the GOI is a gene encoding for any one of a pest toxin, an antisense transcript such as that for patatin or alpha-amylase, ADP-glucose pyrophosphorylase (e.g. see EP-A-0455316), a protease antisense or a glucanase.

The term 'organism' in relation to the present invention includes any organism wherein the promoter can be activated. Preferably the organism is an amylase (e.g. alpha-amylase)

producing organism such as plants, algae, fungi and bacteria, as well as cell lines thereof. Preferably the term means a plant or cell thereof, preferably a dicot, more preferably a potato.

The term 'transgenic organism' in relation to the present invention means an organism comprising either an expressable construct according to the present invention or a product of such a construct. For example the transgenic organism can comprise an exogenous nucleotide sequence (e.g. GOI as herein described) under the control of a promoter according to the present invention; or a native nucleotide sequence under the control of a partially inactivated (e.g. truncated) promoter according to the present invention.

The terms "cell", "tissue" and "organ" include cell, tissue and organ *per se* and when within an organism. For one class/type of promoters according to the present invention the term preferably means a potato stem cell, tissue or organ and a potato sprout cell, tissue or organ for another class/type of promoters according to the present invention it preferably means potato sprout cell, tissue or organ or potato stem cell, tissue or organ.

Preferably the expressable construct is incorporated in the genome of the organism. The term incorporated preferably covers stable incorporation into the genome.

The term 'nucleotide' in relation to the GOI includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA.

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred from one species to another – such as from an *E.Coli* plasmid to a plant cell, or from *agrobacterium* to a plant cell.

Even though the promoters of the present invention are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to put the present invention

into practice. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205–225) and Christou (Agro–Food–Industry Hi–Tech March/April 1994 17–27).

Thus, in one aspect, the present invention relates to a vector system which carries a promoter or construct according to the present invention and which is capable of introducing the promoter or construct into the genome of a plant such as a plant of the family *Solanaceae*, in particular of the genus *Solanum*, especially *Solanum tuberosum*. The vector system may comprise one vector, but comprises preferably two vectors; in the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1–19.

One extensively employed system for transformation of plant cells with a given promoter or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al. (1986), *Plant Physiol.* 81, 301–305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203–208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non–limiting example of such a Ti plasmid is pGV3850.

The promoter or construct of the present invention should preferably be inserted into the Ti–plasmid between the terminal sequences of the T–DNA or adjacent a T–DNA

sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, the vector system of the present invention is preferably one which contains the sequences necessary to infect a plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the promoter or construct may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the promoter or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. See also Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the

transformed cells. The vectors contain for example pBR 332, pUC series, M13 mp series, pACYC 184 etc. In such a way, the construct or promoter can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used a sequence analysis, a restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid. After each introduction method of the desired promoter or construct in the plants further DNA sequences may be necessary. If for example for the transformation, the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells is being intensively studied and is well described in EP 120 516; Hockema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46 and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is another simple technique which may be employed. Typically, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by

subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

In summation therefore the present invention therefore relates to a promoter and, also to a construct comprising the same.

In particular the present invention relates to the use of a promoter for the expression of a GOI in an cell/tissue/organ/ organism such as one or more specific tissues of a plant, in particular a dicot plant such as a potato.

More in particular, in a preferred embodiment, the present invention relates to a partially inactivated (such as truncated) type 1 $\alpha$-amylase promoter.

The present invention also relates to the application of one class of partially inactivated gene promoters to express a GOI specifically in at least the stem or sprout tissue of a dicot – especially a potato plant.

The following sample has been deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St Machar Drive, Aberdeen, Scotland, AB2 1RY, United Kingdom, on 26 August 1994:

DH5alpha-gPAmy 637 (Deposit number NCIMB 40683)

This deposited sample is an *E. Coli* bacterial stock containing the plasmid pBluescript (see Figure 6 for general map thereof) containing the promoter of SEQ. I.D. No. 1 – i.e. the Amy 1 promoter. Details on how the plasmid was formed are given later.

The present invention will now be described only by way of examples, in which reference shall be made to the following Figures, in which:

Figure 1 shows Southern blots for restiction enzyme digests of a clone according

to the present invention (Figures 1A and 1B);

Figure 2 shows a restriction map of a clone according to the present invention;

Figure 3 is a sequence map of a clone according to the present invention;

Figure 4 is the nucleotide sequence of a promoter according to the present invention;

Figure 5 shows the primer sequences for use in the present invention; and

Figure 6 is a plasmid map of pBlueScript KS- (2.96 kb).

In slightly more detail, Figures 1A and 1B are gel patterns of digested DNA of the lamda 637 clone; Figure 2 is a map of the clone lambda 637 and subclones, wherein the darkened boxes are the coding sequences, the empty boxes are the promoter sequences, – — = not sequenced, the shaded boxes represent intron sequences and the shaded and striped boxes represent the transit and leader peptide coding regions; Figure 3 is the sequence map of B2–7 and the individual primers used are indicated above the arrow and correspond to the list given in Figure 5, the arrows show the direction and extent of the individual sequence reactions, the position of the structural gene is shown at the bottom, and E=EcoRI, H=HindIII and B=BamHI; in Figure 4 is the nucleotide sequence of the alpha–amylase promoter sequence and the suggested CAP sites, CCAAT boxes and TATA boxes are underlined; in Figure 5 Uni=T7 primer and Rev = T3 primer.

## MATERIALS AND METHODS

### Plant material

Root tissue were harvested from flowering potato (*Solanum tuberosum*, cv. Saturna) plants. The roots were sliced directly into liquid nitrogen and 10–15g portions were stored at –80°C until use.

Bacterial strains

| | |
|---|---|
| DH5α™ (BRL): | F⁻, endA1, hsdR17($r_k$⁻, $m_k$⁺), supE44, thi-1, λ⁻, recA1, gyrA96, relA1, (argF-lacZYa)U169, σ80dlacZ σM15 |
| JM109(1): | recA1, endA1, gyrA96, thi, usdR17, supE44, relA1, λ⁻σ(lac⁻proAB),[ F', traD36, proAB, LacI$^q$Z σMI5] |
| PLK17 (Stratagene): | hsdR-M+, mcrA-,B-, lac-, supE, gal- |
| LE392 (2,3): | supE44, supF58, hsdR514, galK2, galT22, metB1, trpR55, lac41 |

Phages and plasmids

| | |
|---|---|
| λ EMBL3: | see reference (4) |
| pBS+, pBS-: | see reference (5) |
| pBSK+, pBSK-: | see reference (5) |

Media and plates

L-Broth (LB) medium:

Per litre, 5g of yeast extract, 5g of NZ-amide, 5g of NaCl, 5g of bacto-peptone. Autoclave.

LB-plates:

LB medium plus 15g Bacto agar per litre. Autoclave. Pour into plastic petri dishes (25 ml/dish).

Amp-plates:

As LB-plates plus 35 mg ampicillin per litre after autoclaving.

AXI–plates:

As LB–plates plus 35 mg ampicillin, 120 mg IPTG (isopropylthiogalactoside), 40mg Xgal (dissolved in dimethylformamide) per litre after autoclaving. [Xgal: 5–bromo–4chloro–3indolyle–β–D–galactoside.]

Water:

The water used in Materials and Methods was always distilled and autoclaved before use.

**Isolation of high MW genomic potato DNA**

In order to gain high molecular weight genomic DNA a procedure essentially as described by Fischer and Goldberg (6), was followed. This include first isolation of nuclei followed by preparation of the nuclear DNA.

10–15g Saturna root tissue were ground to a fine powder in liquid nitrogen and homogenized in 100 ml H buffer (1xH buffer(1l): 100ml 10xHB, 250ml 2M sucrose, 10 ml 100mM PMSF, 1ml β–mercaptoethanol, 5 ml Triton X–100, 634ml $H_2O$. Adjust to pH 9.5. Add β–mercaptoethenol just before use. 10xHB: 40mM spermidine, 10mM Spermine, 0.1mM $Na_2$–EDTA, 0.1mM Tris, 0.8mM KCl, adjusted to pH 9.4–9.5 with 10N NaOH. PMSF: phenylmethylsulfonyl fluoride dissolved in ethanol).

The resuspended plant material was filtered through a 70μm nylon filter (Nitex filter, prewetted in 1xH buffer). The resulting filtrate was poured into two centrifuge bottles (Sorvall GSA) and the nuclei pelleted at 4000 r.p.m for 20 min at 4°C. The supernatant was discarded and the pellets were gently resuspended by adding 20 ml 1xH buffer per tube and then swirling the tubes carefully. The nuclei were pelleted again at 4000 r.p.m. for 20 min at 4°C, the supernatant removed and the pellets resuspended gently in 10 ml 1xH buffer. The supernatant was pooled and 20 ml cold lysis buffer (lysis buffer: 2% Sarcosyl, 0.1M Tris, 0.04M $Na_2$–EDTA) was added dropwise while the solution was stirred gently.

Immediately after the last drop of lysis buffer was added, 0.972g CsCl/ml solution was stirred gently into the solution (the solution should now be at room temperature). The resulting solution was centrifuged for 45 min at 10 krpm, 4°C. The supernatants were carefully removed using a pasteur pipet avoiding any protein debris floating on the top or disturbing the pellets. The volume of the supernatants were determined and 0.2 mg ethidium bromide/ml was added.

The DNA solution was then gently poured into quickseal polyallomer tubes, which were then sealed. The tubes were centrifuged in a Beckman VTI 65 rotor at 18°C and 40 k r.p.m. for 38 h.The genomic band was removed under UV-light with a 15-18 gauge needle attached to a 5-ml syringe and poured gently into a 5 ml polyallomer tube. The tube(s) was filled with a 1.57 g/ml CsCl solution in 50mM Tris-Hcl(pH 9.5), 20 mM $Na_2$-EDTA. 75 µl ethidium bromide (5mg/ml) was added/tube. The tubes were centrifuged in the VTI 65 rotor at 18°C and 46 krpm for 17 h. The genomic band was removed under long-wave UV light and the ethidium bromide was extracted with CsCl-saturated isopropanol (7 to 8 times). The CsCl was removed from the DNA by dialysis in TE-buffer (1xTE: 10 mM Tris-HCl, 1 mM $Na_2$-EDTA pH 8.0) at 4°C for 18 h with three changes. The high MW genomic potato DNA was not further precipitated and was kept at 4°C.

Construction of a potato genomic library

High MW genomic potato DNA was prepared from cv Saturna roots as decribed above. The quality of the DNA was tested by restriction enzyme digestion and gel electrophoresis.

The genomic DNA was partially digested with Sau3A and the created fragments (9-23 kb) were inserted into the BamHI site of the λ EMBL3 vector (4). Approximately $1.1x10^6$ independent isolates were plated and amplified to form a permanent library (7).

Plaque hybridization was used to screen the library for α-amylase genes.

Screening of the library

Screening of the potato genomic library was carried out essentially as described by (8,9). The pfu/ml (pfu:plaque forming unit) of the amplified genomic library was determined in duplicate prior to the screening. Infection competent cells (PKL17 or LE392) were prepared by inoculating the cells in 30 ml fresh L-Broth containing 0.2% sucrose and 10 mM $CaCl_2$. The cells were cultivated for 4-5 h at 37°C before 0.1 vol of cold $CaCl_2$ was added and kept on ice until use. 100 µl phages diluted in phagebuffer to give an appropriate number of pfu (1xphagebuffer: 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl) were mixed with 25-100 µl freshly made cells (dependent on the actual number of cells) and incubated at 37°C for 15-20 min. The suspension was mixed with 3 ml warm (42°C) 0.8-1% top agar containing 10 mM $MgCl_2$ and plated out on dry LB plates.

LB plates of 22x22cm (dried for 3-4 h at 37°C) were used for screening of the genomic library. Each plate contained approximately $2x10^5$ plaques, which were mixed with 1 ml of infection competent cells (prepared as above) and incubated for 20 min at 37°C. This mixture was then added to a 25 ml of warm (42-45°C) 0.3% top agarose with 10 mM $MgCl_2$ and the solution was poured onto a fresh dry LB plate. The large LB plates were incubated (not upside down) overnight at 37°C. Phages from the plaques were transferred to Hybond N filters (Amersham) in duplicates. The plates were placed at 4°C for 1 to 2 h to prevent the agarose layer from sticking to the filters. The plates were placed on ice, just before use and they remained on the ice when working with the filters.Two Hybond N filters and a plate were marked for orientation of the filters .

The first filter was laid on the plaques for 45 sec; then floated on denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 7 min, with the phages facing up, then floated on neutralization buffer (0.5 M Tris-HCl (pH 7.4), 3 M NaCl) for 2 times 3 min and finally washed in 2xSSC (1xSSC: 0.15 M NaCl, 0.015 Na-citrate). The filter was air dried and the phage DNA was fixed to the membrane by UV crosslinking. The second filter was laid on the same plate, after the first, for 120 sec and then treated as the first. These filters were used in plaque hybridization following the Hybond N membrane protocol according

to suppliers (Amersham) instructions. X-ray film from both the first and second Hybond N membrane was orientated so that the signals from both filters fitted each other.

The positive plaques were cut with a scalpel (1x1 cm blocks) and submerged in 1 ml phage buffer.

The phage containing tubes were stored airtight (parafilm) at 4°C after 2-3 drops of chloroform has been added. The plaque containing plates (22x22 cm) were stored by placing a piece of soaked (chloroform) filterpaper in the lid. The plates were also stored airtight at 4°C with the plaques facing up. Further purification of the positive plaques were done by plating dilutions of the stock tube (containing the 1x1 cm block) with freshly prepared cells and plate them on round LB plates with 1% warm (42°C) top-agar and 10 mM $MgCl_2$.

New filter prints were made with Hybond N following the procedure outlined above with the 22x22 cm plates. Plaques which gave positive signal were isolated by sticking the tip of a pasteur pipette though the plate and transfer it to 500 µl phagebuffer. New series of dilutions were made, plated and the respective filters hybridized until the positive plaques were purified. The phages were stored airtight, at 4°C either in the 500 µl phagebuffer with 1 drop of chloroform, or as phages isolated from a plate lysate.

The plate lysate stock was made as decribed by (9).

Isolation of recombinant λ DNA

Large-scale preparations followed the method decribed in (14), which included binding the recombinant phage DNA on a CsCl gradient. Two versions (A,B) of a small-scale preparation were used as follows:

A) LE392 cells were inoculated in LB with 0.2% maltose and 10 mM $MgCl_2$ and grown O/N at 37°C. The cells were pelleted by centrifugation for 10 min, at 4°C in a Sorvall centrifuge, and resuspended gently in 1 volume of cold 10 mM $MgSO_4$. The cells were

stored at 4°C until use. Five single plaques from a plate were transferred to 500 µl phagebuffer and allowed to stand for 2-2$^1/_2$ h at 4°C.

After votex of the tube 100 µl of the liberated phages were mixed with 200 µl freshly prepared LE392 cells. Alternatively 50-100 µl liberated phages from aplate lysate were mixed with the cells. Phages and cells were incubated for 20 min at 37°C and then added to a prewarmed (37°C) 25 ml LB with 20 mM $MgSO_4$ and 30 mM Tris-HCl pH 7.5 and incubated, shaking O/N at 37°C. A further 10 ml prewarmed LB with 20 mM $MgCl_2$ and 30 mM Tris-HCl pH 7.5 was added and the mix incubated for 1-2 h shaking at 37°C. After clear lysis (eventuel a few drops of chloroform was added to help) and the solution was centrifuged at 8000 r.p.m. for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged again if necessary to remove cell debris. The recombinant λ DNA was then purified using a Qiagen column following the suppliers instructions (10).

B) The procedure was as under A) until after the first centrifugation of the O/N culture. The supernatant was transferred to a new tube and DNase was added corresponding to 1 µg/ml.

The solution was incubated 30 min at 37°C and then 1 volume of cold 20% PEG, 2 M NaCl mixed in phagebuffer was added and the mixture was incubated 1 h on ice. The phages were pelleted by centrifugation for 20 min, 4°C at 10 krpm. The PEG pellet was resuspended in 400 µl phagebuffer and transferred to an eppendorf tube. 1 µl of RNase (10 mg/ml) is added and the tube incubated for 30 min at 37°C. Then 8 µl 0.25 M $Na_2$-EDTA, pH 8.0 and 4 µl 10% SDS was added, the tube was incubated a further 15 min at 68°C.

The mixture was allowed to gain room temperature and then an equal phenol saturated with TE-buffer (1xTE: 10 mM Tris pH 7.5, 1 mM $Na_2$-EDTA) was used to extract the DNA. A equal mixture of saturated phenol-chloroform was used to extract the upper aqueous phase and a final chloroform extraction was done. The upper phase was transferred to a new tube and the solution was made 0.3 M Na-acetate and 2-3 vol cold ethanol was added.

The precipitation of the DNA was accomplished by storing at O/N at -20°C, centrifuging for 5 min and resuspend the pellet in 50-100 µl TE-buffer. The amount and quality of the recombinant phage DNA was tested by restrictions enzyme digest and agarose (0.8-1%) gel electrophoresis (11).

Preparation of plasmid DNA

The plasmid preparation was as described in EP-B-0470145. In particular, small scale preparation of plasmid DNA was performed as follows.

Bacterial strains harbouring the plasmids were grown overnight in 2 ml L-Broth (LB) medium with ampicillin added (35 µg/ml). The operations were performed in 1.5 ml Eppendorf tubes and centrifugation was carried out in an Eppendorf centrifuge at 4°C.

The cells from the overnight culture were harvested by centrifugation for 2 min., washed with 1 ml 10 mM Tris-HCl (pH 8.5), 50 mM EDTA and centrifuged for 2 min. The pellet was suspended in 150 µl of 15% sucrose, 50 mM Tris-HCl (pH 8.5), 50 mM EDTA by vortexing. 50 µl of 4 mg/ml lysozyme was added and the mixture was incubated for 30 min. at room temperature and 30 min. on ice. 400 µl ice cold $H^2O$ was added and the mixture was kept on ice for 5 min, incubated at 70-72°C for 15 min. and centrifuged for 15 min.

To the supernatant was then added 75 µl 5.0 M Na-perchlorate and 200 µl isopropanol (the isopropanol was stored at room temperature), and the mixture was centrifuged for 15 min. at 4°C. The pellet was suspended in 300 µl 0.3 M Na-acetate and 2-3 vol. cold ethanol was added.

Precipitation was accomplished by storing at either 5 min. at -80°C or O/N at -20°C, centrifuging for 5 min., drying by vacuum for 2 min. and redissolving the pellet in 20 µl $H_2O$. The yield was 5-10 µg plasmid DNA.

Large scale preparation of plasmid DNA was accomplished by simply scaling up the small scale preparation ten times. Working in 15 ml corex tubes, all the ingredients were scaled up ten times. The centrifugation was carried out in a Sorvall cooling centrifuge at 4°C. Only changes from the above will be mentioned in the following. After incubation at 70–72°C, the centrifugation was for 30 min. at 17,000 rpm. After adding isopropanol and after adding cold ethanol, the centrifugation was for 15 min. at 17,000 rpm.

The final plasmid DNA pellet was then suspended in $H_2O$ and transferred to an Eppendorf tube and then given a short spin to remove debris. The supernatant was adjusted to 0.3 M Na–acetate and 2–3 vol. cold ethanol were added. The pellet was resuspended in 40 µl $H_2O$. The yield was usually 20–28 µg plasmid DNA.

To obtain very pure plasmid DNA, 200–300 µg of isolated plasmid DNA from the upscaled method were banded on a CsCl gradient. Solid CsCl was mixed with $H_2O$ (1:1 w/v) and 0.2 mg/ml ethidium bromide was added. The solution was poured into a quick–seal polyallomer tube and the plasmid DNA, mixed with solid CsCl (1:1 w/v). The tube was filled, sealed and centrifuged in a Beckman VTI 65 rotor at 15°C, 48,000 rpm for 16–18 hours. The centrifuge was stopped by without using the brake. The banded plasmid DNA was withdrawn from the tubes using a syringe and the ethidium bromide was extracted with CsCl–saturated isopropanol 7–8 times. The CsCl was removed by dialysis in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA for 48 hours with three changes of buffer. The DNA was precipitated by adjusting to 0.3 M Na–acetate and adding 2–3 vol.cold ethanol.

The small scale plasmid preparation from *E. coli* was usually followed by a LiCl precipitation to remove RNA from the DNA solution. The small scale prepared plasmid DNA was dissolved in 100 µl destilled water. 1 vol of 5M LiCl was added and the mixture incubated at –20°C for 30 min followed by centrifugation at 12,000 rpm. for 15 min, 4°C. The supernatant was transferred to a new eppendorf tube and 2 vol TE buffer or water was added. Precipitation with 2.5 vol of 96% ethanol was accomplished by storing either 10 min. at –80°C, or O/N at –20°C. The DNA was precipitated by centrifuging for 15 min. 12,000 rpm ,at 4°C, drying by vacuum for 2 min and redissolving

in 18 µl of TE or water.

Restriction enzyme digestion

The protocol followed was that outlined in EP-B-0470145. In particular, all restriction endonucleases were from Biolabs , Amersham or Boehringer Mannheim and were used according to the supplier's instructions. 1 unit of enzyme was used to 1 µg of DNA and incubation was for 2 hours. The buffer was changed in double digestions, by changing the volume or by adding the necessary ingredient according to the enzyme instructions.

Labelling of DNA

A random primed DNA labelling kit (Boehringer Mannheim) was used according to the suppliers instructions. Briefly, 2 µl DNA fragment (25-50 ng) is mixed with 8 µl $H_2O$ and incubated at 95°C for 10 min to denature the DNA. Spin shortly and place on ice. Then add 1 µl dGTP, dATP and dTTP of each, 2 µl reactionsmix and 5 µl (approx. 50 µCi $dCTP^{32}$). 1 µl Klenow enzyme starts the reaction and the tube is incubated at 37°C for 30 min. Then place on ice. The labelled DNA fragment was purified using an ELUTIP column (Schleicher & Schuell). The column was prepared by prerunning (gravity) it with 3 ml high salt buffer (1.0 M NaCl, 20 mM Tris-HCl (pH 7,5), 1.0 mM EDTA), followed by 5 ml low salt buffer (0.2 M NaCl, 20 mM Tris-HCl (pH 7,5), 1.0 mM EDTA). 250 µl low salt was added to the labelling tube and the entire solution was laid on the prepared column. Then the column was washed with 2x400 µl low salt followed by 3x200 µl high salt. The eluted radioactive probe was then heat denatured and used in hybridization.

Southern transfer and Hybridization

The DNA fragments to be transferred were fractionated on non-denaturing agarose gels (109) and transferred to either Hybond™ N or Hybond™ N+, positively charged nylon membrane (Amersham Life Science) by Southern blotting (12,13).

Hybridization to the Hybond™ N nylon membranes was followed according to the supplier's instructions (13).

Preparation of vectors

The preparation of vectors was as decribed in EP-B-0470145 as follows: Vectors (pBS-/+ or pBSK-/+) were digested with one or two restriction enzymes, extracted twice with saturated phenol (the phenol was first mixed with 0.1 M Tris-HCl, then mixed twice with TE-buffer (10 mM Tris-HCl, pH 8, 1 mM $Na_2$-EDTA)) and once with chloroform and precipitated with 0.3 M Na-acetate and 2.5 vol cold ethanol. The pellet was rinsed in 70% cold ethanol and dissolved in $H_2O$, giving a concentration of 25-50 ng/$\mu$l. The vectors were tested for background before use (self-ligation with and without T4-DNA-ligase). If necessary the vector was treated with Alkaline phosphatase (Boehringer Mannheim) as described by the supplier. After such a treatment the resulting pellet was resuspended in $H_2O$ to give a final concentration of 10 ng/$\mu$l.

Ligation

The phage DNA or plasmid comprising a fragment to be subcloned was digested with one or more restriction enzymes and run in either a 5% acrylamide gel or an appropriate agarose gel. The fragment to be subcloned was isolated from the gel either by electroelution as decribed in (109) or using a GENECLEAN II Kit (BIO 101 Inc., La Jolla, California) following the suppliers instructions.

Various ratios of fragment to vector were used (from 2:1 to 5:1, based on the number of molecules) in the ligation reaction. 1 $\mu$l (10-100 ng) of a solution containing the vector was combined with the fragment, 1 $\mu$l of T4-ligation buffer (10x(20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.6 mM ATP, 10 mM dithiotheitol)) and 1 $\mu$l of T4-DNA ligase (Boehringer Mannheim) were added to a mixture of fragment and vector to a total volume of 10 $\mu$l. The reaction was incubated at 15°C O/N if the ligated DNA fragments had sticky ends. If the DNA had blunt ends, the incubation was at room temperature for 1 hour. The ligation mixture was stored at -20°C if not used immediately, usually 5 $\mu$l of

the ligation mix was used for transformation.

Preparation of competent *E. coli* cells and transformation

This was done according to the protocols laid down in EP-B-0470145 as follows:

JM109 cells (or DH5$\alpha$) were inoculated in 4 ml L-Broth made to 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The cells were grown O/N at 37°C. 1 ml of the O/N culture was added to 40 ml prewarmed (37°C) LB medium (with 10 mM $MgSO_4$ and 10 mM $MgCl_2$). The culture was shaken at 250-275 rpm for 1 to 2 h until the $OD_{450}$ reached 0.8-0.9. The cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was gently resuspended in 30 ml of cold 0.1 M $CaCl_2$, another centrifugation pelleted the cells again and they were then resuspended in 15 ml of cold 0.1 M $CaCl_2$. The suspension was placed on ice for 20 min followed by a centrifugation as before. Finally, the cells were gently resuspended in 3 ml of cold 0.1 M $CaCl_2$ and placed on ice for at least 1 h before they were ready to use for transformation (14).

5 $\mu$l of ligation mix was combined with 95 $\mu$l of cold sterile TCM (10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.2 ml of the competent cells.

The mixture was allowed to stand for at least 40 min on ice, then 5 min at 37°C (or 2 min at 42°C). The solution was transferred to 0.8 ml of L-Broth, 10 mM MgSO4, 10 mM $MgCl_2$, and incubated for 45 min at 37°C and then plated out on 5 AXI or other plates (as e.g. Amp-plates) at 0.2 ml/plate. The plates were allowed to stand 10 min before being inverted and incubated O/N at 37°C.

They were then stored in plastic bags upside down at 4°C.

Purification of primers following synthesis on a DNA Synthesizer

The primer was synthesized on a polystyrene support column (Applied Biosystems, 393 DNA/RNA Synthesizer) and was eluted from the column with $NH_4OH$.

The column was broken open and 1.5 ml $NH_4OH$ was added to the polystyrene material in a small glass tube. The mixture was incubated at 85°C for 1 hour followed by 5 min on ice. The supernatant containing the single stranded DNA was transferred to eppendorf tubes, and the $NH_4OH$ was evaporated in a vacuum centrifuge for at least 3 h.Pellet was resuspended in 200 µl destilled water and precipitated with 550 µl ethanol and 20 µl sodium acetate. The pellet was resuspended in 200 µl water and precipitation with ethanol and sodium acetate was repeated. Finally the pellet was resuspended in 100 – 200 µl destilled water and the $OD_{260nm}$ was measured by a Gene Quant RNA/DNA calculator (Pharmacia) of single stranded DNA is calculated. An $OD_{260nm}$ of 1 corresponds approx. to 33 µg/ml single stranded DNA.

Subcloning and sequencing

Purified λ DNA was digested with appropiate restrictions enzymes and the generated fragments were isolated from agarose gels using a GeneClean Kit (BIO 1 Inc., La Jolla, California) according to the suppliers instructions.

Genomic DNA fragments (or fragments obtained from plasmids) were ligated into the polylinker region of the BlueScribe vector pBS-/+ (or pBSK-/+, Stratagene). After transforming an *E. coli* strain with the ligated plasmid the recombinant subclones could be selected by plating on AXI plates (they will be white and the nonrecombinant clones will be blue when the vector is a pBlueScribe plasmid,(5)).

Plasmid DNA from putative subclones were digested with appropiate restriction enzymes, subjected to gelelctrophoresis and after Soutern blotting, hybridized with an appropiate labelled DNA probe, to verify the origin of the inserted fragment.

The generated pBS genomic DNA subclones were then sequenced according to the plasmid preparation protocol outlined in EP-B-0470145. In this regard, the plasmid (double stranded template) to be sequenced was purified by the plasmid small scale preparation method. The DNA was denatured in 0.2 M NaOH (5 min at room temperature) the mixture was neutralised by adding 0.4 vol of 5 M ammonium acetate (pH

7.5) and then precipitated with 4 vol. of cold ethanol (5 min at −80°C). The pellet was rinsed with 70°C cold ethanol and resuspended in 10 $\mu$l $H_2O$.

Sequencing was accomplished with a Sequenase™ DNA Sequencing Kit from United States Biochemical Corp., following the sequencing Protocol enclosed in the kit (Sequenase™ :Step by Step Protocols for DNA sequencing with Sequenase, 3rd Edition, United States Biochemical Corporation PO Box 22400 Cleveland Ohio 44122).

The following modifications were however made to the suggested protocol. Instead of adding DTT, Labelling mix and $^{35}$SdATP to the annealed DNA mix, 4 ml of $^{35}$Sequetide (DuPont) was added.

In addition to T3 and T7 primers (Stratagene) a whole range of other primers generated on a DNA synthesizer (Applied Biosystems, 392 DNA/RNA Synthesizer) were used. 0.5 pmol of primer was used to sequence 1 pmol of plasmid. The primer sequences are shown in the attached Figure 5. The sequencing reactions were subjected to electrophoresis on 6 % or 8 % denaturing polyacrylamide gels for 1 to 4 hours at 40 W, then dried by a gel drier and autoradiographed for 3-48 hours at room temperature.

The denaturing sequencing gels were made from pre-mixed polyacrylamide solutions , Gel-Mix 6 and Gel-Mix 8 (GIBCO BRL, Life technologies, Inc) according to the manufacturers instructions.

**Isolation of α-amylase genomic clones**

Several cDNA clones encoding α-amylase from potato (*Solanum tuberosum*) had previously been isolated (described in EP-B-0470145). The plasmid pAmyZ6 (EP-B-0470145) encoding a partial α-amylase was digested with ClaI and SmaI. This creates two fragments of approx. 500 bp covering the α-amylase coding sequence and untranslated 3'end. Both fragments were used as probes (see "DNA labelling" in Materials and Methods) to screen the genomic poatato λ DNA library (see "construction of a potato genomic library" in Materials and Methods). Screening of approx. $1.6 \times 10^6$

phages were carried out as decribed in Materials and Methods. Twenty four positive plaques were isolated and λ DNA (see "Materials and Methods") was made from 8 of them. A SalI digest (release the inserted genomic fragment from the λ arms) showed that the 8 clones contain genomic fragments ranging from approx 10.5 kb to 13.5 kb in size.

The clone gPAmy637 was chosen for further analysis by Southern blotting and hybridizations.

The α-amylase clones pAmyZ6 and pAmyZ1 (see EP-B-0470145 for a detailed description) contain sequences encoding an α-amy 1 type of α-amylase. They are however, not identical and using both for hybridizations to Southern blots which contain EcoRI, HindIII and BamHI digests of the gPAmy637 clone, revealed a slightly different hybridization pattern between them (see Figure 1A and 1B).

Prominent hybridizing bands, (called B1 and B2 in the BamHI digested lane, H1 and H2 in the HindIII digested lane and, E1 and E2 in the EcoRI digested lane, Figure 1) from all three digests of gPAmy637 were subcloned into pBS and pBSK vectors predigested with appropiate enzymes.

Plasmid DNA isolated from transformed *E.coli* cells (see Material and Methods) were digested with appropiate restriction ezymes and used for Southern analysis using the insert of pAmyZ6 (it was cut out of the pBSK plasmid using AccI and SacI) as probe, to verify their origin.

A BamHI map of the gPAmy637 λ clone is shown in Figure 2. This map covers approx. 2/3 of the genomic DNA insert in the clone (insert size approx. 13.5 kb). Using various fragments of the gPAmyZ6 and the gPAmyZ1 cDNA clones (5' and 3' fragments) as probes to Southern blots of digests of both gPAmy637 and another λ clone gPAmy651, it was possible to map more than one gene encoding the α-amy 1 type α-amylase. The position of both an α-amy 1 type gene, corresponding to the pAmyZ1 cDNA clone and another α-amy 1 type pseudogene, corresponding to the pAmyZ6 cDNA clone are shown in Figure 2.

Sequencing covering most of this region of the gPAmy637 λ clone verified the identity of the genes. The sequenced region included the B1 (partially) and B2 subclones (B1:B1-5 and B2:B2-7) and in addition the H1 and H2 subclones (H1:H1-H and H2:H2-J) see figure 2 for their mapping positions.

Subcloning a genomic fragment containing an α-amylase promoter from potato

The B2-7 genomic fragment (see Figures 1 and 2) of 3763 bp was subcloned from gPAmy637 into a dephosphorylated BamHI site of a pBKS- vector (see Materials and Methods). This subclone contains the ATG initiation codon, 1669 bp downstream of it covering approx. half of the structural gene and 2094 bp upstream covering the promoter region of the α-amy 1 type α-amylase gene. As shown by others (e.g. see 15-22) the sequence region upstream of the ATG initiation codon covering about 1000-1500 bp include the entire promoter, enough to mediate transcription of the gene at the right time and place.

Sequence of an α-amylase promoter

The sequence of the BamHI subclone (B2-7) isolated from gPAmy637 was determined (see Materials and Methods). This covers in total 3763 bp and the sequence map is shown in figure 3. The individual primer used is indicated in Figure 3 and corresponds to the list given in figure 5. The DNA sequence of the 2094 bp promoter region is shown in Figure 4. The promoter sequence was compared with published sequences in the EMBL database (using a PC-gene program from IntelliGenetics, Inc., California) and no sequence with significant overall homology was found.

Putative TATA- and CAAT- boxes are underlined in Figure 4 and possible CAP sites are also indicated. Their positions correspond to the positions they are found in other eucaryotic polymerase II promoters (23-24).

An expression analysis of the α-amylase gene of the present invention revealed that the α-amylase type 1 gene(s) is weakly expressed in potato sprouts. However, it is more

strongly expressed in potato stems. This type is not found in leaves, tuber or root tissues.

In summation, the applicability of the promoters of the present invention is widespread. With the promoters it is possible to direct the expression of proteins into different tissues in the potato plant. It would also be possible to direct the expression of proteins into different tissues in other dicot plants.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

NAME OF APPLICANTS: DANISCO A/S
BUSINESS ADDRESS: Langebrogade 1
DK-1001 Copenhagen K
Denmark

TITLE OF INVENTION: Promoter

SEQ. ID. NO. 1

SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA
ORIGINAL SOURCE: *Solanum Tuberosum*
SEQUENCE LENGTH: 2094
SEQUENCE:

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        10         20         30         40
ATTAAGGGGA GCATAAGTGC AGCTCAGAAA TTCACACCTG

        50         60         70         80
ATATTTTCCC AAAGCCCTCA AAAATGTGAA CAAATCTGCT

        90        100        110        120
AAAATGTCAG TCAGAAGGAC TGTTCTTTTA GGTTTTCTTC

       130        140        150        160
TCTCGAGTCA CGAAATCAGA TAATATGATA AGAAATTATG

       170        180        190        200
GAGGATTTAT AATGTATCTG TCTGTTCTTA GGTATAATTA

       210        220        230        240
TGTGTTCCTT TATGATGTAG TAATGGAATT CTGGGCTTAT

       250        260        270        280
ATTAAAGGAA CTGAATATAA ATGTTCGCAT TTTAACTGCG

       290        300        310        320
GAGACTTCGA GTTAGAGCCT TATAATTATG TCTTATCATT

       330        340        350        360
TTATACTGAG ATCATATTAC AGATGATGAA AGCTGACATT

       370        380        390        400
GCATTAGTTA TTCTGTTTTA TACAAGTCAT GTAACTGCTG

       410        420        430        440
CTTGTGAGTT GTGACTGTAA GATAAATTGA TTCAGCCTTC

       450        460        470        480
TGTGGCATTA GCGGAGATCT GATTATACTC TCATCGTCTT
```

```
       490         500         510         520
ATCTAAGTTG CTCATGCAAC TTTGTCCTTG ATAGTTGGCT

       530         540         550         560
AATACTACAA CTGGAATTAA GTGTAGTTAT TCGAAATCTC

       570         580         590         600
TGTTGGAAGT TGCTAAGTGC TTAAGTGCTG GTTATTGTAA

       610         620         630         640
ACCCCATCCG AGTTATTATA CAGCATCTGG CTGATGAAAT

       650         660         670         680
GCTGCTCATT TGCAATGGTG ACATAACCAA ATGTTAGTAA

       690         700         710         720
AACATACTAG CTGGTTGAAT GTTAGATGAT TGTTCAACGT

       730         740         750         760
TACATCTCAC AGAAACCTTA TTATGGATTG ACATGTTAGT

       770         780         790         800
TGATCCGAAA GATCCTTCTT TTAAATGCCA AAGCTTGTTA

       810         820         830         840
CAGATTTGAG GAGTTCTTTT ACTTTCTTTT GTTATATCTA

       850         860         870         880
TTTCCCATTC ATTTTGACGT TCAGCCTCAC AGATGTTGTC

       890         900         910         920
ATACTTAGAA ATGTGCGTAT ATATATAGAG AGAGAGAGAT

       930         940         950         960
AGAGTGAAAT GATTATATAG TCGAAGATTA CGAAACTTGA

       970         980         990        1000
CATTGAGACA TCTGTGATTG TTTGAAATTT ATGTATATAT

      1010        1020        1030        1040
CTGTAGCATT AGAAACTATA AGAGTTGTTA GCTTCACTTG

      1050        1060        1070        1080
TCTTATTGTT GTGCTCAAAG CAACTTCATC ATACAGTATG

      1090        1100        1110        1120
GTTTTTATAT GCTCTTCCAT TATCACCGAA CCTTATGATT

      1130        1140        1150        1160
ATGTGTACGA GCTTATAATA TTACTGATGG TGATTCAGTA

      1170        1180        1190        1200
TTATGATTAT GTCCTCCATT AATTATTCTG TTTCATACAA
```

```
      1210        1220        1230        1240
GTCGTGTAAT TTGCTGTTTG TGATTGTACG ATAAATTGAT

      1250        1260        1270        1280
TCAACCTTCT GCGGTGTTGG TTGAAGTTCA AGTAAATTAG

      1290        1300        1310        1320
CTTTATTTAT CATAGTAGCA TTTGATTATT GATGCTCTGT

      1330        1340        1350        1360
AGCTAATGAT AAGCCATTGA AGGGAAGCAG AAATGGTAAA

      1370        1380        1390        1400
GCTTTCTAAA ATGAATCTAC GAATGGATGA TAAAGTTAAT

      1410        1420        1430        1440
GAATATTGTT GATACTTCTG CAATCAGATT ATGAGTTACT

      1450        1460        1470        1480
GAGTCTACTG TTTTTTAAGC CTGTTTCAGA TGATCGATCA

      1490        1500        1510        1520
TCAACAACAA CATATTCAGT GTAGTAGACA TGATCGATCA

      1530        1540        1550        1560
CTTTCTAATT TTCGATTATG CACCCTCTTT TCTCCAATTT

      1570        1580        1590        1600
GGTCGTCTTC TTTTTTTCAT GATGTCACTG AATTATTCTC

      1610        1620        1630        1640
TGGTCGTCCC CACCATTCAG GAAGTCACTT CGAGCATAAT

      1650        1660        1670        1680
GTGAAAACAT CCACATTTTT CAAATCCAGC AGAATTTTCA

      1690        1700        1710        1720
TCAAACGGGG TTCAACATTT ACTACATGTA TACACTCTGA

      1730        1740        1750        1760
AGTCTGAATC CACTAATTCT AGATGGTGCA TCTGTGCCCC

      1770        1780        1790        1800
CACACTTGTG AAAGCTTATT CTCAATTTTT TATTTTCCAA

      1810        1820        1830        1840
CAACTTGAAT TCAGACCACA CAACTCCCGT GTCTTGTACG

      1850        1860        1870        1880
GTCAGCATCT GAGTGGAGAA CTCAATTAAG TGACTTTAAC

      1890        1900        1910        1920
GTCGAGTTCT ATAGTAAACA ACCCCTATAT CTTTTTTCAA
```

1930 1940 1950 1960
GCATGTTAAG ATTGCGAACA CACTGAAATT TCCAGGTCGT

1970 1980 1990 2000
TAATCTTGTA CCCAGTGTGT GTACTTTTAA AAAAAAAAGT

2010 2020 2030 2040
CAGTTTTTTA GTCTCTAAAA CACATTTAAA TAGAGTTTAT

2050 2060 2070 2080
TTGCCATCTT TTGTTCCTCA TACTAGACTT CGGAGTCAAC

2090
ACAACACAAC AACA

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12, lines 18-23

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution (*including postal code and country*)

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

| Date of deposit | Accession Number |
|---|---|
| 26 August 1994 | NCIMB 40683 |

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

For receiving Office use only

☒ This sheet was received with the international application PCT/EP 95/02195

Authorized officer O. GORGE

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☐ This sheet was received by the International Bureau on:

Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. A promoter comprising the sequence shown as Seq.I.D.No. 1 or a variant, homologue or fragment thereof.

2. A promoter comprising the sequence shown as Seq.I.D.No. 1 or a variant, homologue or fragment thereof but wherein at least a part of the promoter is inactivated.

3. A promoter comprising a sequence that is a truncated version of the sequence shown as Seq.I.D.No. 1.

4. A construct comprising the promoter according to any one of claims 1 to 3 fused to a GOI.

5. An expression vector comprising the invention according to any one of claims 1 to 4.

6. A transformation vector comprising the invention according to any one of claims 1 to 5.

7. A transformed cell comprising the invention according to any one of claims 1 to 6.

8. A transgenic organism comprising the invention according to any one of claims 1 to 7.

9. A transgenic organism according to claim 8 wherein the plant is a potato plant.

10. Use of a promoter as defined in claim 1 or claim 2 or claim 3 to direct expression of a GOI in sprout or, preferably, stem cell, tissue or organ of a plant.

FIG 1

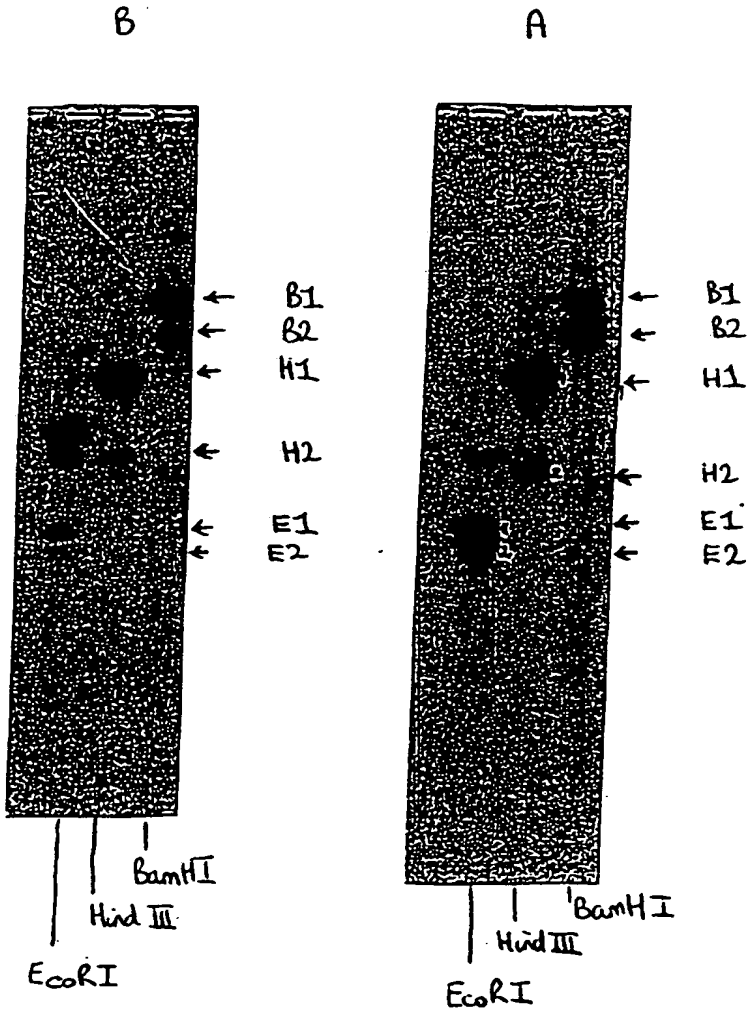

B
A
B1
B2
H1
H2
E1
E2
BamHI
Hind III
EcoRI
B1
B2
H1
H2
E1
E2
BamHI
Hind III
EcoRI

Fig 2.

Clone λ 637 and subclones

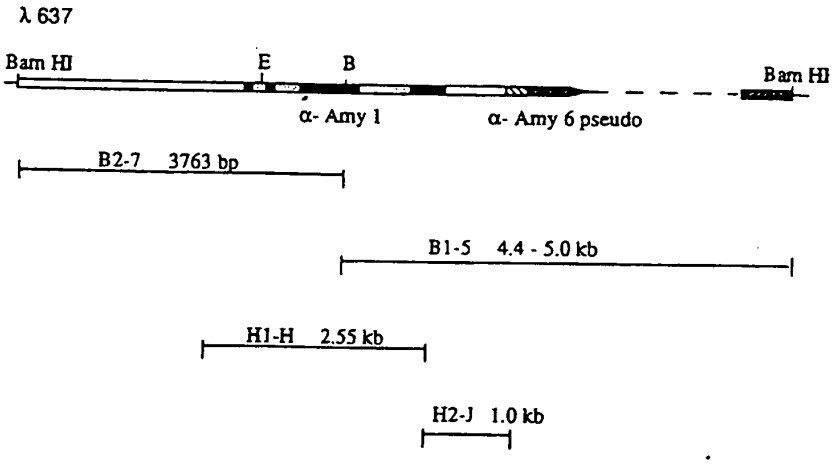
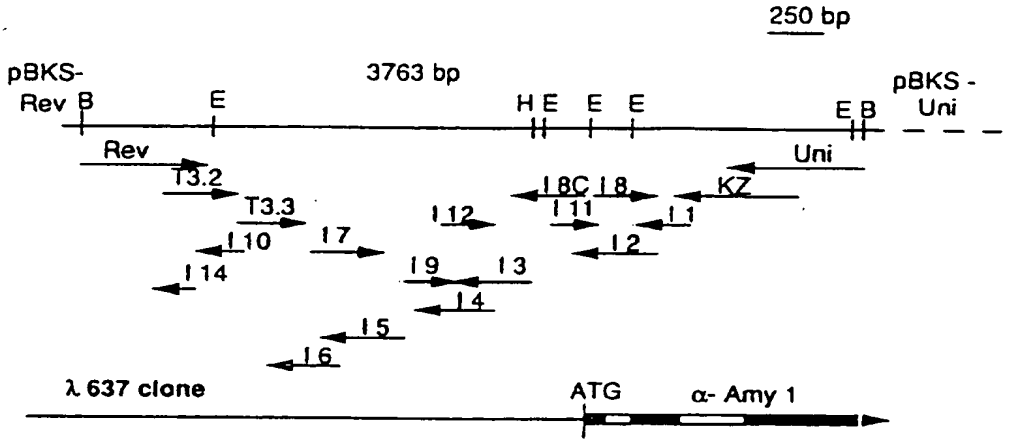


Fig 3

Sequence map of B2-7




Intron
Exon
Leader/ transit peptid
Untranslated/ promotor sequence

The individual primer used are indicated above the arrow and correspond to the list given in figure 5. The arrows shows the direction and extent of the individual sequence reactions. The position of the structural gene is shown at the bottom.
E = Eco RI H = Hind III B = Bam HI

FIG 4

-2094 ATTAAGGGGAGCATAAGTGCAGCTCAGAAATTCACACCTGATATTTTCCC

-2044 AAAGCCCTCAAAAATGTGAACAAATCTGCTAAAATGTCAGTCAGAAGGAC

-1994 TGTTCTTTTAGGTTTTCTTCTCTCGAGTCACGAAATCAGATAATATGATA

-1944 AGAAATTATGGAGGATTTATAATGTATCTGTCTGTTCTTAGGTATAATTA

-1894 TGTGTTCCTTTATGATGTAGTAATGGAATTCTGGGCTTATATTAAAGGAA

-1844 CTGAATATAAATGTTCGCATTTTAACTGCGGAGACTTCGAGTTAGAGCCT

-1794 TATAATTATGTCTTATCATTTTATACTGAGATCATATTACAGATGATGAA

-1744 AGCTGACATTGCATTAGTTATTCTGTTTTATACAAGTCATGTAACTGCTG

-1694 CTTGTGAGTTGTGACTGTAAGATAAATTGATTCAGCCTTCTGTGGCATTA

-1644 GCGGAGATCTGATTATACTCTCATCGTCTTATCTAAGTTGCTCATGCAAC

-1594 TTTGTCCTTGATAGTTGGCTAATACTACAACTGGAATTAAGTGTAGTTAT

-1544 TCGAAATCTCTGTTGGAAGTTGCTAAGTGCTTAAGTGCTGGTTATTGTAA

-1494 ACCCCATCCGAGTTATTATACAGCATCTGGCTGATGAAATGCTGCTCATT

-1444 TGCAATGGTGACATAACCAAATGTTAGTAAAACATACTAGCTGGTTGAAT

-1394 GTTAGATGATTGTTCAACGTTACATCTCACAGAAACCTTATTATGGATTG

-1344 ACATGTTAGTTGATCCGAAAGATCCTTCTTTTAAATGCCAAAGCTTGTTA

-1294 CAGATTTGAGGAGTTCTTTTACTTTCTTTTGTTATATCTATTTCCCATTC

-1244 ATTTTGACGTTCAGCCTCACAGATGTTGTCATACTTAGAAATGTGCGTAT

-1194 ATATATAGAGAGAGAGAGATAGAGTGAAATGATTATATAGTCGAAGATTA

-1144 CGAAACTTGACATTGAGACATCTGTGATTGTTTGAAATTTATGTATATAT

-1094 CTGTAGCATTAGAAACTATAAGAGTTGTTAGCTTCACTTGTCTTATTGTT

-1044 GTGCTCAAAGCAACTTCATCATACAGTATGGTTTTTATATGCTCTTCCAT

- 994 TATCACCGAACCTTATGATTATGTGTACGAGCTTATAATATTACTGATGG

- 944 TGATTCAGTATTATGATTATGTCCTCCATTAATTATTCTGTTTCATACAA

- 894 GTCGTGTAATTTGCTGTTTGTGATTGTACGATAAATTGATTCAACCTTCT

-844 GCGGTGTTGGTTGAAGTTCAAGTAAATTAGCTTTATTTATCATAGTAGCA

-794 TTTGATTATTGATGCTCTGTAGCTAATGATAAGCCATTGAAGGGAAGCAG

FIG 4 continued

```
-744  AAATGGTAAAGCTTTCTAAAATGAATCTACGAATGGATGATAAAGTTAAT
-694  GAATATTGTTGATACTTCTGCAATCAGATTATGAGTTACTGAGTCTACTG
-644  TTTTTTAAGCCTGTTTCAGATGATCGATCATCAACAACAACATATTCAGT
-594  GTAGTAGACATGATCGATCACTTTCTAATTTTCGATTATGCACCCTCTTT
-544  TCTCCAATTTGGTCGTCTTCTTTTTTTCATGATGTCACTGAATTATTCTC
-494  TGGTCGTCCCCACCATTCAGGAAGTCACTTCGAGCATAATGTGAAAACAT
-444  CCACATTTTTCAAATCCAGCAGAATTTTCATCAAACGGGGTTCAACATTT
-394  ACTACATGTATACACTCTGAAGTCTGAATCCACTAATTCTAGATGGTGCA
-344  TCTGTGCCCCCACACTTGTGAAAGCTTATTCTCAATTTTTTATTTTCCAA
-294  CAACTTGAATTCAGACCACACAACTCCCGTGTCTTGTACGGTCAGCATCT
-244  GAGTGGAGAACTCAATTAAGTGACTTTAACGTCGAGTTCTATAGTAAACA
-194  ACCCCTATATCTTTTTTCAAGCATGTTAAGATTGCGAACACACTGAAATT
-144  TCCAGGTCGTTAATCTTGTACCCAGTGTGTGTACTTTTAAAAAAAAAAGT
 - 94  CAGTTTTTTAGTCTCTAAAACACATTTAAATAGAGTTTATTTGCCATCTT
 - 44  TTGTTCCTCATACTAGACTTCGGAGTCAACACAACACAACAACA
                                                      -1
      +1
      ATG
```

Suggested CAP sites , CCAAT-boxes and TATA -boxes are underlined.

FIG 5

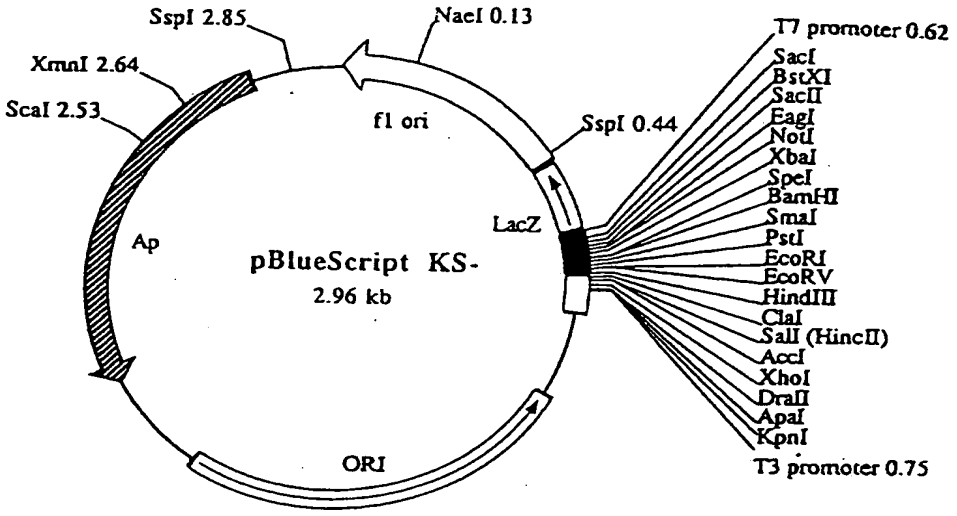
Primer sequences.

| Name | 5' - Sequence - 3' |
|---|---|
| I 1 | CACCGAGGCTT GAGCGGG |
| I 2 | GAGGAGCAACA GAGTGAG |
| I 3 | GTCTGAATTCA AGTTGTGG |
| I 4 | CGATCATCTGA AACAGGC |
| I 5 | GGTTCGGTGAT AATGGAAG |
| I 6 | CTCCTCAAATC TGTAACAAGC |
| I 7 | GGAATTAAGTG TAGTTATTCG |
| I 8 | CCTCATACTAG ACTTGGAGTC |
| I 8 C | GACTCCAAGTC TAGTATGAGG |
| I 9 | GAGACATCTGT GATTGTTTG |
| I 10 | CCGCTAATGCC ACAGAAGGC |
| I 11 | GTAAACAACCC CTATATC |
| I 12 | GCCATTGAAGG GAAGCAG |
| I 14 | GCCCAGAATTC CATTACTAC |
| T3-2 | CTTGATGCAGG GGTGCGATG |
| T3-3 | GAAAGCTGACA TTGCATTAG |
| KZ | GGGGCAGGTTC AAAGTCC |

Uni = T7 primer
Rev = T3 primer

Figure 5

FIG 6


SspI 2.85
NaeI 0.13
T7 promoter 0.62
XmnI 2.64
SacI
BstXI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI (HincII)
AccI
XhoI
DraII
ApaI
KpnI
ScaI 2.53
f1 ori
SspI 0.44
LacZ
Ap
pBlueScript KS-
2.96 kb
ORI
T3 promoter 0.75

# INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/EP 95/02195

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | WO-A-90 12876 (DANISCO A/S) 1 November 1990<br>see pages 5-8, 20-21, 33, 37 and 40, Example 29 and Claims.<br><br>& EP-B-0 470 145<br>cited in the application<br>--- | 1,4-10<br>2,3 |
| Y | PLANT CELL,<br>vol. 4, 1992<br>pages 1435-1441,<br>F. GUBLER AND J.V. JACOBSEN;<br>'Gibberellin-responsive elements in the promoter of a barley high-pI alpha-amylase gene'<br>see abstract and discussion.<br>---<br>-/-- | 2,3 |

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 January 1996 | 20 February 1996 ( 20.02.96) |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+ 31-70) 340-3016 | Authorized officer<br>Yeats, S |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Internat ' Application No
PCT/EP 95/02195

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ' | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | J. BIOL. CHEM., vol. 269, 1994 pages 17635-17641, M.-T. CHAN ET AL.; 'Novel gene expression system for plant cells based on induction of alpha-amylase promoter by carbohydrate starvation' see abstract and introduction. --- | 1 |
| A | PLANT MOL. BIOL., vol. 19, 1992 pages 891-901, P.J. RUSHTON ET AL.; 'Aleurone nuclear proteins bind to similar elements in the promoter regions of two gibberellin-.related alpha-amylase genes' see abstract and introduction. --- | 1 |
| A | EP-A-0 375 092 (INSTITUT FUR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH) 27 June 1990 see claims. & CA-A-2 006 454 cited in the application ----- | 1 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

L. ..mation on patent family members

Internat ' Application No
PCT/EP 95/02195

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9012876 | 01-11-90 | AU-B- | 638248 | 24-06-93 |
| | | AU-B- | 5531890 | 16-11-90 |
| | | DE-D- | 69008172 | 19-05-94 |
| | | DE-T- | 69008172 | 20-10-94 |
| | | EP-A- | 0470145 | 12-02-92 |
| | | ES-T- | 2053189 | 16-07-94 |
| | | IE-B- | 63067 | 22-03-95 |
| EP-A-375092 | 27-06-90 | DE-A- | 3843627 | 05-07-90 |
| | | CA-A- | 2006454 | 21-06-90 |
| | | JP-A- | 2283276 | 20-11-90 |
| | | US-A- | 5436393 | 25-07-95 |